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Drought controls on H_2O_2 accumulation, catalase (CAT) activity and *CAT* gene expression in wheat

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Abstract

Plants co-ordinate information derived from many diverse external and internal signals to ensure appropriate control of gene expression under optimal and stress conditions. In this work, the relationships between catalase (CAT) and H_2O_2 during drought in wheat (*Triticum aestivum* L.) are studied. Drought-induced H_2O_2 accumulation correlated with decreases in soil water content and CO_2 assimilation. Leaf H_2O_2 content increased even though total CAT activity doubled under severe drought conditions. Diurnal regulation of *CAT1* and *CAT2* mRNA abundance was apparent in all conditions and day/night *CAT1* and *CAT2* expression patterns were modified by mild and severe drought. The abundance of *CAT1* transcripts was regulated by circadian controls that persisted in continuous darkness, while *CAT2* was modulated by light. Drought decreased abundance, and modified the pattern, of *CAT1 and CAT2* mRNAs. It was concluded that the complex regulation of CAT mRNA, particularly at the level of translation, allows precise control of leaf H_2O_2 accumulation.

Key words: Catalase, diurnal cycle, drought, hydrogen peroxide, *Triticum aestivum* L.

Introduction

Drought stress is a complex syndrome involving not only water deprivation but also nutrient limitation, salinity, and oxidative stresses. Moreover, levels of light that are optimal for photosynthesis in well-watered plants become excessive in plants suffering water deprivation. Photosynthesis is particularly sensitive to water deficit because the stomata close to conserve water as available soil water declines. Stomatal closure deprives the leaves of carbon dioxide and photosynthetic carbon assimilation is decreased in favour of photorespiratory oxygen uptake. The process of stomatal closure and the enhancement of flux through the photorespiratory pathway increase the oxidative load on the tissues as both processes generate reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2). Hydrogen peroxide is also generated as a secondary messenger in abscisic acid (ABA)-mediated stomatal closure (Pei *et al.*, 2000). In photorespiration, H_2O_2 is produced at very high rates by the glycollate oxidase reaction in the peroxisomes (Noctor *et al.*, 2002). Moreover, superoxide production by the photosynthetic electron transport chain (via the Mehler reaction) is exacerbated by drought (Noctor *et al.*, 2002).

Plants respond to diverse environmental signals in order to survive stresses such as drought (Pastori and Foyer, 2002). Strategies to minimize oxidative damage are a universal feature of plant defence responses. Hydrogen peroxide is eliminated by catalases (CAT) and ascorbate peroxidases (Chen and Asada, 1989; Scandalios *et al.*, 1997). These enzymes rapidly destroy the vast majority of H_2O_2 produced by metabolism, but they allow low steady-state levels to persist presumably to maintain redox signalling pathways (Noctor and Foyer, 1998).

Catalase is essential for the removal of H_2O_2 produced in the peroxisomes by photorespiration (Noctor *et al.*, 2000). Catalase activities decrease under conditions that suppress photorespiration, such as elevated CO_2 (Azevedo *et al.*, 1998). The importance of CAT in photosynthetic cells is demonstrated by observations in CAT-deficient mutants (Kendall *et al.*, 1983) and in transformed tobacco in which the major leaf CAT isoform is decreased by antisense technology (Takahashi *et al.*, 1997; Willekens *et al.*, 1997). When such plants are placed in conditions favouring

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high rates of photorespiration (low CO_2, high light, warm temperatures), photosynthesis is inhibited, the foliar antioxidant system is perturbed, and necrotic lesions appear on the leaves. Moreover, induction of defence-related proteins is observed, both locally in the necrotic regions and in leaves which do not suffer necrosis (Takahashi *et al.*, 1997; Willekens *et al.*, 1997). However, the CAT protein is susceptible to photoinactivation upon exposure to high light intensities (Shang and Feierabend, 1999) and leaf CAT activities have been shown to decline in certain stress conditions (Hertwig *et al.*, 1992). There is now considerable evidence to show that CAT is one of the most rapidly turned over proteins in leaf cells particularly in stress conditions. Catalase gene expression and translation, as well as CAT protein turnover, are regulated in a complex manner that is far from resolved.

Plant catalases are encoded by a small gene family, usually composed of three isozyme genes which exhibit fairly complex spatial and temporal patterns of expression throughout the plant life cycle (Scandalios *et al.*, 1997; Willekens *et al.*, 1997). The wide diversity of plant *CAT* sequences has led to some discrepancies in the isozyme correlation in phylogenetic trees. This has been investigated and a model for the evolutionary divergence of monocot and dicot *CAT* genes has been proposed (Iwamoto *et al.*, 1998). The presence of a G-box or ABRE (ABA responsive) element in the maize *CAT1* promoter allows increased expression in response to exogenous ABA and osmotic stress (Guan and Scandalios, 2000). An antioxidant responsive element (ARE) is also present in the promoters of *CAT1* and *CAT3* underlying the important protective role of *CAT* in response to oxidative stress (Polidoros and Scandalios, 1999). In addition, the expression of some *CAT* genes is under circadian control (Zhong and McClung, 1996; Polidoros and Scandalios, 1998). The aims of the present study were: (i) to determine the effect of drought on H_2O_2 content and CAT activity in relation to photosynthesis in the leaves of a major crop species, wheat; (ii) to examine the effects of stress on the day/night abundance of mRNAs encoding the two major leaf *CAT* isoforms, *CAT1* and *CAT2*, and (iii) to establish relationships between leaf H_2O_2 content, CAT activity, and *CAT1* and *CAT2* transcripts under optimal and stress conditions.

Materials and methods

Plant material and growth conditions

Wheat (*Triticum aestivum* L.) plants were sown in individual pots and grown in a glasshouse at 20 °C and 550 μmol quanta m^{-2} s^{-1} at canopy height, as described by Noctor *et al.* (2002). Four varieties were used in this study: the spring varieties 'Canon' and 'Cadenza', the model cultivar 'BobWhite', and the winter variety 'Buster'. The maximum soil-water content recorded in these experiments for well-watered conditions was 60%. Five-week-old plants of the four varieties were subject to mild and severe drought. Mild drought delivered a gradual water deprivation of 16 plants for up to 6 d, a point equivalent to a reduction to 40% of the original soil water content at day 6. Plants subject to mild drought were well-watered 12 h before the commencement of the treatment and provided with a lower reservoir of water throughout the experiment. Severe drought was carried out by rapid desiccation of 20 plants over 2–4 d to a final soil water content of 15–20% at the final harvest point. In these conditions, plants were well-watered 12 h before the treatment and the plants thereafter had no water supply throughout the duration of the drought period. Well-watered plants irrigated from above and below, of the same age were used as controls: 10 plants at the beginning of the treatment (control) and eight plants at the end of the treatment (developmental control). Three independent experiments were performed using third leaves from each individual plant, which were excised each day at 09.00 h, quickly frozen in liquid N_2, and stored at −80 °C until extraction. Third leaves from three individual plants and three independent experiments were used for molecular analyses. CO_2 assimilation was measured by an infrared gas exchange analyser (model WA-225-MK3, ADC, Hoddesdon, Hertfordshire, UK). Stomatal conductance was estimated from water vapour measured by infrared gas analysis as above. Studies on circadian rhythm were carried out in two independent experiments using 5-week-old 'Cadenza' plants, which were grown and treated as described above. Third leaves collected from six control plants, six mild-stressed plants, and six severely-stressed plants at different times during the day/night cycle, were frozen at −80 °C until extraction.

Analysis of CAT gene expression by RT-PCR

Gene sequences from wheat or *Arabidopsis* were obtained from the GenBank database. The accession numbers and primers designed for the sequences analysed are: *Arabidopsis Actin-1* M20016; *ATACT-F*, 5′-GAGAAGATGACTCAGATC-3′ and *ATACT-R,* 5′-ATCCTTCCTGATATCGAC-3′; wheat *CAT1* E16461, *CAT1-F*, 5′-ACTACGACGGGCTCATG-3′ and *CAT1-R*: 5′-GGAGCTGAGA-CGGCTTC-3′; wheat *CAT2* X94352, *CAT2-F*, 5′-CCTTAATCAG-CAGGGATG-3′ and *CAT2-R*, 5′-AGATAGAACACGCGGAG-3′. PCR reactions were performed using a programmable Robocycler at annealing temperatures of 44 °C for *Actin* and 46 °C for *CAT*. For an accurate comparison and quantification of the transcript levels, the exponential phase of PCR amplification was determined by establishing the number of PCR cycles where the products exhibit an exponential phase: 35 cycles for *Actin* PCR products and 30 cycles for *CAT* PCR products. The identity of all PCR products was confirmed by sequencing analysis at the Department of Biochemistry of Oxford University.

H_2O_2 and catalase activity measurements

Leaf H_2O_2 contents and catalase activity were measured on the third leaf of control and drought-treated wheat plants. H_2O_2 was determined as in Veljovic-Jovanovic *et al.* (2002). Catalase activity was assayed as described by Vanacker *et al.* (2000) using 0.1 M HEPES pH 6.5, 10 mM $MgCl_2$, and 5 mM EDTA.

Results

Photosynthesis, leaf catalase activity, H_2O_2 accumulation and drought

The relationships between soil water content and leaf photosynthetic CO_2 assimilation rates were similar in the three varieties 'Canon', 'Cadenza' and 'Buster' (Fig. 1). Severe inhibition of photosynthesis was observed only when the soil water content decreased to below 30%. Leaf water potentials and stomatal conductance showed similar trends with respect to drought in all varieties (Fig. 2). Based on these results, 'Cadenza' was chosen for further investigation

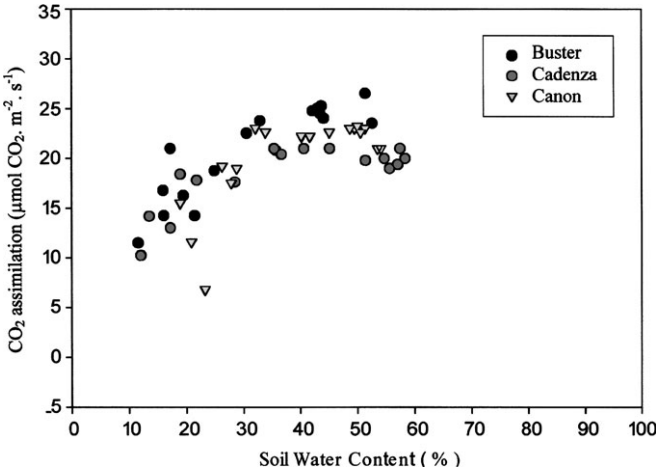

Fig. 1. Drought-induced inhibition of photosynthesis in wheat. Leaf CO_2 assimilation rates and soil water contents were measured after 5-week-old plants that experienced either progressive mild drought (up to a 60% decrease in soil water content) or severe drought (up to an 80% decrease in soil water content). Values are the means of three independent series of experiments.

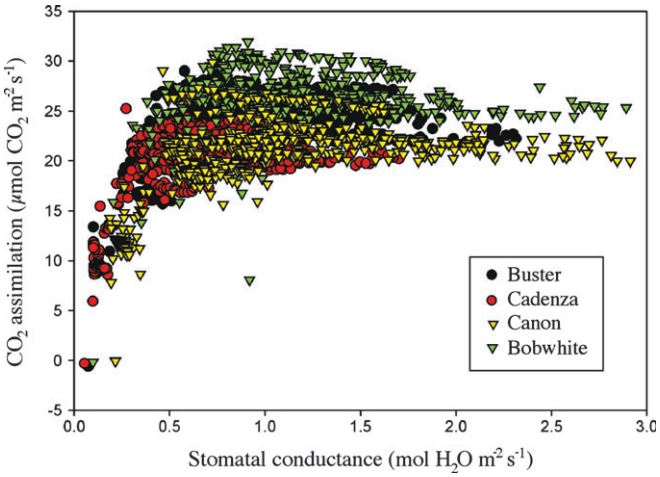

Fig. 2. The relationship between CO_2 assimilation and stomatal conductance in response to drought. CO_2 assimilation rates and stomatal conductances were measured in 5-week-old plants.

and two experimental regimes (mild drought and severe drought) were identified from the data presented in Figs 1 and 2. 'Mild drought' is classified as exposure to gradual decreases in soil water to as low as 40% over 6 d. Severe drought is defined as exposure to rapid decreases in soil water to as low as 15–20% over 2–4 d. CO_2 assimilation rates together with soil water content and stomatal conductance, were estimated in each of the following experiments: they did not vary from the pattern shown in Figs 1 and 2, and are hence not included here. Total leaf catalase activity was significantly increased only in response to severe drought, when the soil water content was less than 20% (Fig. 3). Leaf H_2O_2 concentrations, on the other hand, increased progressively as soil water content decreased (Fig. 4).

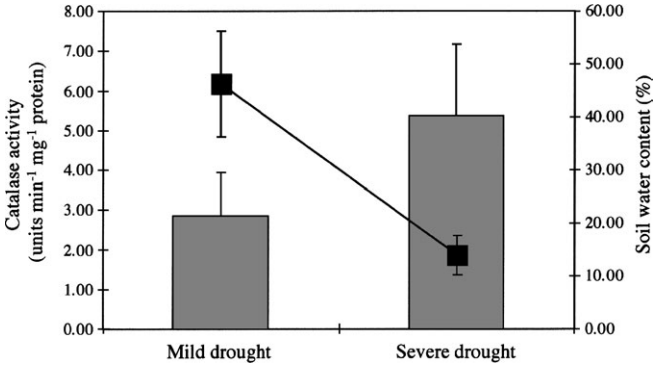

Fig. 3. The effect of decreasing soil water content (grey bars) on leaf catalase activities (black squares). Plants were grouped in two sets: (i) 16 plants were subjected to mild drought in a range of 36–55% SWC; (ii) 20 plants were subjected to severe drought in a range of 11–18% SWC. One unit is defined as 1 mmol min^{-1} mg^{-1} protein. Catalase activity in leaves of water-replete plants (60% SWC) was 3.75 U min^{-1} mg^{-1} protein.

Drought-induced shifts in the diurnal cycle of CAT *gene expression*

The abundance of *CAT1* and *CAT2* transcripts fluctuated over the day/night cycle in well-watered plants and this pattern was modified by drought (Fig. 5). *Catalase1* mRNA levels were highest early in the light period (after 4 h) and lowest after 1 h dark (Fig. 6A). *Catalase2* mRNA levels were also highest in the light, with a maximum after 12 h illumination (Fig. 6D). Like *CAT1, CAT2* transcripts decreased rapidly following the transition to darkness with minimal values obtained after 1 h in the dark (Fig. 6D). To investigate whether these changes represented a diurnal rhythm in gene expression the fluctuations of *CAT* transcript levels were analysed over the 24 h light/dark cycle maintaining one set of plants in continuous darkness (Fig. 6A, D). The pattern of *CAT1* expression in leaves maintained in complete darkness was almost identical to the pattern observed in control leaves maintained in the regular day/night conditions during the first 24 h. By contrast, the pattern of abundance of *CAT2* transcripts was markedly shifted upon exposure to continuous dark (Fig. 6A, D). This suggests that *CAT1* is regulated by circadian controls while *CAT2* is modulated by light/dark.

The pattern of *CAT1* transcript abundance was modified by water deficit, with diurnal changes significantly shifted in time and intensity under mild and severe drought conditions (Fig. 6B, C). In plants subjected to mild drought the pattern of *CAT1* expression was basically similar to that of the water-replete control with two maximal peaks at 09.00 h and 17.00 h. However, the *CAT1* peak of expression at 17.00 h was strongly enhanced under mild drought compared with water-replete conditions, peak intensity being increased by about 2.5-fold. A different pattern of *CAT1* transcript accumulation was observed in plants subjected to severe water stress (Fig. 6C). In this case, only one peak of *CAT1* expression was observed. This occurred at 13.00 h, 4 h earlier than the peak observed in

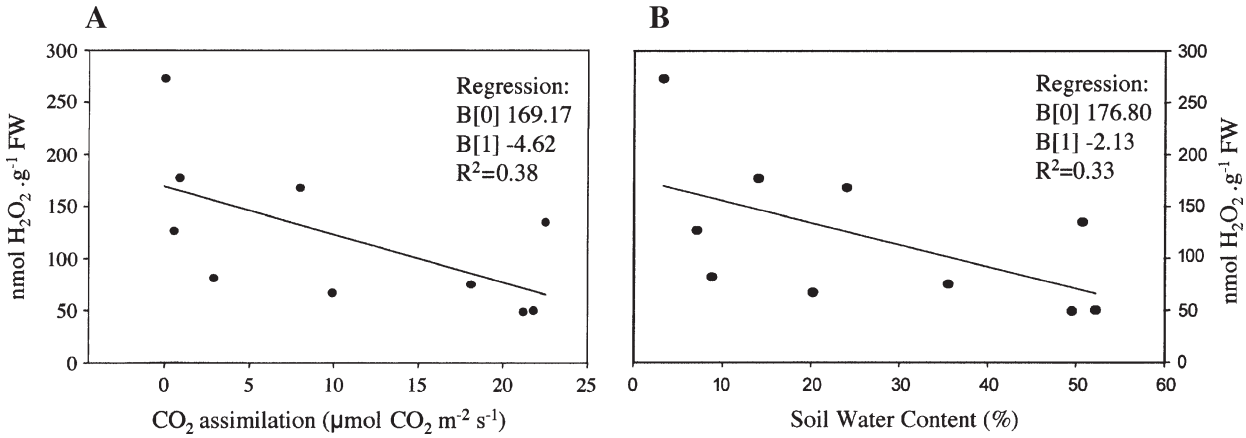

Fig. 4. The relationship between leaf H_2O_2 content and CO_2 assimilation (A) and soil water content (B).

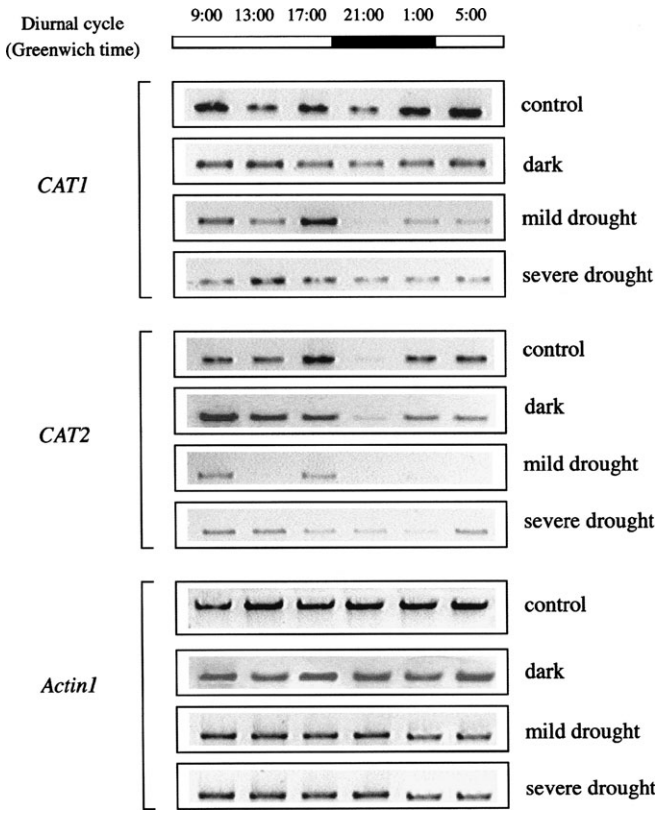

Fig. 5. Drought-mediated effects on the diurnal cycle of catalase (*CAT1 and CAT2*) transcript accumulation in wheat leaves (var. 'Cadenza'). Leaf samples were harvested at different time points during the day/night cycle indicated as white and black bars, respectively. Five-week-old plants were grown either under water-replete conditions with the normal day/night cycle (control), or in continuous darkness (dark), or with mild drought (40% soil water content; see Fig. 1) or severe drought (20% soil water content; see Fig. 1) with the normal day/night cycle. The figure shows results typical of those obtained in three independent experiments. *Actin I* was used as an internal control.

mild drought (Fig. 6B). Moreover, the increase in the peak of *CAT1* transcripts was lower than that observed under mild drought (Fig. 6B, C).

The pattern of *CAT2* transcript abundance was also modified by drought (Fig. 6D–F). In well-watered leaves, two significant peaks of *CAT2* expression were observed, one occurring during the day at 17.00 h and the second was observed at night (after 5 h darkness). The pattern of *CAT2* expression was also clearly different under mild and severe drought (Fig. 6E, F). Under mild drought, two significant peaks of *CAT2* expression were observed during the day, at 09.00 h and 17.00 h. By contrast, the intensity of change in *CAT2* expression was greatly decreased under severe drought with a small increase at 13.00 h (Fig. 6E, F).

Drought effects on photosynthetic performance in the light

Leaf CO_2 assimilation began immediately when the light was switched on in all cases, with little indication of an induction period. Photosynthesis rapidly attained stable maximal rates, that were maintained throughout the light period in water-replete leaves (Fig. 6G). By contrast, leaves exposed to water stress showed a marked induction period before CO_2 assimilation reached maximal rates (Fig. 6H, I). Moreover, overall photosynthesis performance was lower in water-stressed leaves than water-replete controls (Fig. 6H, I). For example, CO_2 assimilation rates were between 25 and 30 µmol m^{-2} s^{-1} in water-replete leaves (Fig. 6G) and leaves exposed to mild drought (Fig. 6H), whereas maximal values no higher than 15 µmol m^{-2} s^{-1} were measured under 'severe' drought conditions (Fig. 6I). Moreover, a progressive decline in the rate of CO_2 assimilation during the light period was found in leaves subjected to both mild and severe water stress (Fig. 6H, I).

Discussion

Water availability is one of the major factors limiting plant productivity in the field. Photorespiration is greatly enhanced as a result of water deficits because the stomata close and CO_2 assimilation is inhibited. The effects of

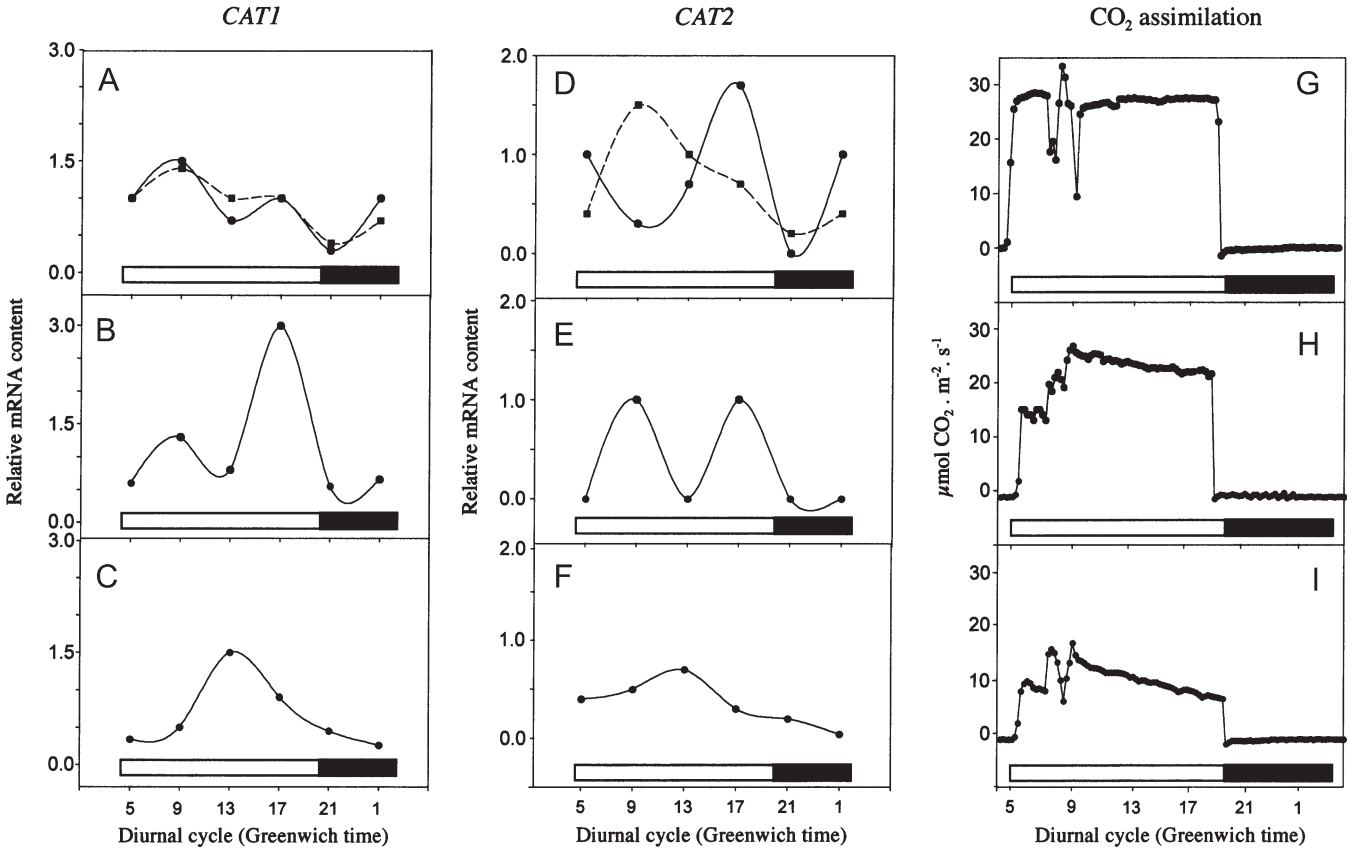

Fig. 6. A comparison of the time-course of day/night fluctuations in the abundance of *CAT1* transcripts (left column, A, B, C), *CAT2* transcripts (middle column, D, E, F) and photosynthesis rates (right column, G, H, I). Leaf samples were harvested from 5-week-old plants grown under water-replete conditions with the normal day/night cycle (control, A, D, G), in continuous darkness (dashed lines, A, D), or experiencing mild drought (B, E, H) or severe drought (C, F, I) with the normal day/night cycle. The day/night cycle is indicated as white and black bars, respectively. Relative mRNA values were calculated as the ratio *CAT1/Actin I* (A, B, C) and *CAT2/Actin I* (D, E, F). The figure shows results typical of those obtained in three independent experiments.

limiting CO_2 availability (as occurs when stomata close) on rates of wheat leaf H_2O_2 formation, extrapolated from measured rates of photosynthesis and photorespiration have previously been calculated (Noctor *et al.*, 2002). The robustness of plant cells to H_2O_2 and other oxidants is due to effective controls of oxidant levels by a versatile leaf antioxidative system. The general responses of wheat leaf antioxidants to drought are well documented (Smirnoff and Colombe, 1988; Pastori and Trippi, 1993; Menconi *et al.*, 1995; Bartoli *et al.*, 1999). This, together with an understanding of the crucial role of CAT in removal of H_2O_2 formed by photorespiration led to the present study being focused on the drought-induced regulation of catalase and H_2O_2 accumulation. The results presented here show that (i) H_2O_2 accumulates in wheat leaves in response to drought as water is depleted from the soil; (ii) CAT activity significantly increases only under severe drought; (iii) day/night *CAT1* and *CAT2* expression patterns are modified by mild and severe drought; and (iv) drought-induced changes in CO_2 assimilation rates are comparable to those obtained in other studies.

Endogenous circadian rhythms in the activities of free radical detoxification enzymes are found in all living organisms (Hardeland, 2000). These are often associated with rhythms in metabolism, such as regulation of photosynthesis, that generate cycles in cellular redox state. Environmental signals and oxidative stress modify circadian controls (Hardeland, 2000), thus drought-induced changes in *CAT* expression patterns are not surprising. Similar responses have been reported in other drought-responsive genes (Carpenter *et al.*, 1994; Cellier *et al.*, 2000; Thompson and Corlett, 1995). It is shown that the expression of *CAT1* and *CAT2* is modulated by light in wheat as it is in maize and *Arabidopsis (*Acevedo *et al.*, 1991; McClung, 1997). Wheat *CAT1* expression shows characteristics of circadian control, as indicated by the persistence of the rhythm in darkness, and its expression pattern is equivalent to the clock-regulated maize *CAT3* and *Arabidopsis CAT2* genes (Acevedo *et al.*, 1991; Zhong *et al.*, 1994). Instead, *CAT2* expression does not appear to be clock-regulated in wheat, similar to results reported for maize *CAT2* and *Arabidopsis CAT1* (Acevedo *et al.*, 1991; McClung, 1997). Catalase

isozyme gene correlations between species, and a model for the evolutionary divergence of *CAT* genes, have been proposed by Iwamoto *et al.* (1998). The results presented here agree with those of previous studies on *CAT* gene expression (Guan and Scandalios, 2000). Hence *CAT1* and *CAT2* transcript abundance is highest in the light and broadly correlated with H_2O_2 formation in photorespiration. However, the extent of CAT mRNA accumulation in leaves of plants experiencing drought is lower than that observed in well-watered plants in the light. The increase in CAT activity observed in leaves experiencing water deficits cannot therefore be explained by enhanced transcription. Translation of *CAT* mRNAs depends on the supply of the methyl group donors, from glycine and serine, whose production is greatly enhanced by photorespiratory carbon flow (Schmidt *et al.*, 2002). Catalase protein synthesis is therefore linked to the photosynthetic and photorespiratory pathways (Schmidt *et al.*, 2002).

It might be asked why regulation of CAT has this high degree of complexity. The answer must reside in the requirement of a precise control of leaf H_2O_2 levels. The role of H_2O_2 in stress-induced damage has long been recognized, but it is now also generally accepted that H_2O_2 is an integral component of cell signalling cascades (Mittler, 2002; Vranova *et al.*, 2002) and an indispensable second messenger in biotic and abiotic stress situations (Green and Fluhr, 1995; Pastori and Foyer, 2002). More recently H_2O_2 has been reported to be intimately involved in a wide range of hormone-dependent developmental signalling processes, as well as in cell wall cleavage and associated cell wall growth (reviewed by Foyer and Noctor, 2003). Hydrogen peroxide is also considered to fulfil a signalling role in guard cells through the control of stomatal closure (Schroeder *et al.*, 2001; Kohler *et al.*, 2003). It was concluded that CAT regulation serves to limit excessive H_2O_2 accumulation while allowing essential signalling functions to occur.

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